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of

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for

CLONING AND CHARACTERIZATION OF THE flbA GENE OF H. PYLORI, PRODUCTION OF AFLAGELLATE STRAINS

CLONING AND CHARACTERIZATION OF THE flbA GENE OF H.PYLORI. PRODUCTION OF AFLAGELLATE STRAINS

Helicobacter pylori (also designated as H.pylori) is a Gram-negative bacterium which, to date, has been found exclusively on the surface of the mucosa of the stomach in man.

In common with most bacteria, H.pylori is sensitive to a medium which is at acid pH but, nevertheless, is able to tolerate acidity in the presence of physiological concentrations of urea (Marshall et al. (1990) Gastroenterol. 99: 697-702). By hydrolysing the urea to form carbon dioxide and ammonia, which are released into the microenvironment of the bacterium, the H.pylori urease enables the bacterium to survive in the acidic environment of the stomach. Recently, studies carried out on animal models have provided data suggesting that the urease is an important factor in the colonization of the gastric mucosa (Eaton et al. (1991) Infect. Immun. 59: 2470-2475). The urease is also suspected of causing injury, either directly or indirectly, to the gastric mucosa.

Currently, Helicobacter pylori (H.pylori) is recognized as being the etiological agent of antral gastrites, and appears to be one of the cofactors required for the development of ulcers. Furthermore, it

appears that the development of gastric carcinomas may be associated with the presence of H.pylori.

In order to develop novel sensitive and specific means for detecting in-vitro infections due to bacteria of the Helicobacter pylori species, the inventors have been taking an interest in the system for regulating the mobility of these bacteria.

With this aim in view, they have been interested in different modifications of the H.pylori strains, modifications which did not affect the recognition of these bacteria by sera from infected patients but which nevertheless rendered it possible to avoid obtaining reactions of the "false positive" type, in particular with bacteria of the Campylobacter family, for example Campylobacter jejuni.

Furthermore, the inventors observed that it was possible, if need be, for the modified bacteria which were obtained to be employed in constructing immunogenic compositions or compositions used for vaccination. In this respect, the invention proposes, in particular, live attenuated bacterial strains.

In a first step, the inventors identified and isolated the gene flbA which is involved in the regulation of the biosynthesis of the flagella of H.pylori and, as a consequence, in the regulation of the mobility of the bacterium. The biosynthesis of the flagella comprises synthesizing flagellins A and B and synthesizing the sheath. The flbA gene regulates both the synthesis of flagellins A and B and the synthesis of the sheath which contains these flagellins. The inventors established that the flbA gene was also important in that it regulated the biosynthesis of the anchoring protein of the bacterium, also termed the "hook".

The invention therefore relates to a nucleotide sequence from the flbA gene regulating the biosynthesis of the proteins of the Helicobacter pylori flagella, characterized in that it is able to hybridize, under conditions of high stringency, with a probe

corresponding to a nucleotide fragment from H.pylori which has been amplified using two oligonucleotides having the following sequences:

OLF1bA-1: ATGCCTCGAGGTCGAAAAGCAAGATG (SEQ ID NO:1)

OLF1bA-2: GAAATCTTCATACTGGCAGCTCCAGTC (SEQ ID NO:2)

or able to hybridize, under conditions of high stringency, with these oligonucleotides.

Such a sequence can be obtained by the steps of:

- screening a genomic library containing the chromosomal DNA of an H.pylori strain with a probe corresponding to a nucleotide fragment from H.pylori which has been amplified using two oligonucleotides having the following sequences:

OLF1bA-1: ATGCCTCGAGGTCGAAAAGCAAGATG (SEQ ID NO:1)

OLF1bA-2: GAAATCTTCATACTGGCAGCTCCAGTC (SEQ ID NO:2)

or able to hybridize, under conditions of high stringency, with these oligonucleotides,

- recovering the DNA sequences which hybridize with the said probe,

- subcloning the DNA sequences which have been obtained in an appropriate vector of the plasmid type and selecting those modified vectors which hybridize, under conditions of high stringency, with the probe corresponding to the DNA fragment from H.pylori which has been amplified using oligonucleotides OLF1bA-1 and OLF1bA-2,

- sequencing the DNA fragments contained in the plasmid vectors which hybridize with the abovementioned probe and determining the open reading frame contained in these fragments.

Advantageously, these DNA fragments will be used to reconstitute the coding sequence of the flbA gene, corresponding to an open reading frame comprising approximately 2196 nucleotides.

The genomic library containing the chromosomal DNA of H.pylori can be obtained from any H.pylori strain. A cosmid library may also be prepared from the chromosomal DNA of H.pylori.

An example of a strain which can be used for constructing this library is the strain N6, which was deposited in the NCIMB on 26 June 1992 under No. NCIMB40512.

The two oligonucleotide primers which are used for preparing the probe which is intended for hybridizing the sought-after DNA which is present in the H.pylori DNA library are selected from the conserved regions of the various proteins of the LcrD/FlbF family.

The two oligonucleotide primers, OLFlbA-1 and OLFlbA-2, enabled a fragment to be amplified which was usable as a probe and which was of 130 base pairs, having the following sequence:

ATG CCA GGA AAG CAA ATG GCG ATT GAT GCG GAT TTA AAT TCA ~~GGA~~ GGC A CTT ATT GAT GAT AAG GAA GCT AAA AAA CGG CGC GCC GCT CTA AGC CAA GAA GCG GAT TTT TAT GGT GCG ATG GAT GGC GCG TCT AAA TTT (SEQ ID NO:3)

The conditions of high stringency referred to above are the following: the hybridization is carried out at 42°C in the presence of 50% formamide in a 2xSSC buffer containing 0.1% SDS (1xSSC corresponds to 0.15 M NaCl plus 15 mM sodium citrate - pH 7.0). The washings are carried out at 68°C, for example twice during a period of one hour, using 2xSSC plus 0.1% SDS.

A nucleotide sequence which is particularly interesting in accordance with the invention is the sequence of the flbA gene corresponding to the sequence of nucleotides (SEQ ID NO:6) depicted in Figure 2, or to a nucleotide sequence which hybridizes, under conditions of high stringency, with the abovementioned sequence.

According to another embodiment of the invention, the nucleotide sequence which is the subject-matter of the present application is characterized in that it encodes a protein having the amino acid sequence (SEQ ID NO:7) depicted in Figure 2 or an amino acid sequence possessing the same regulatory properties, with regard to the biosynthesis of the flagellar proteins of H.pylori, as the abovementioned sequence.

The invention also relates to a nucleotide sequence which corresponds to the previous definitions and which is modified by deletion, substitution or insertion of bases or of a fragment of a nucleotide sequence, such that:

- either the flbA gene is no longer expressed in a host cell,

- or the expression of the flbA gene in a host cell does not enable the A and B flagellins or the sheath which contains them to be biosynthesized and, if this is the case, does not enable the H.pylori anchoring protein or the hook, to be synthesized.

The modification to which the nucleotide sequence of the invention is subjected should be such that it is irreversible and, in particular, that it remains irreversible when this sequence is recombined with the flbA gene which is present in a bacterium which is transformed with a nucleotide sequence which is modified in this manner. This recombination is, for example, of the "double crossing over" type. Preferably, the modification of the nucleotide sequence should not involve any substantial modification - after replacement, by this modified sequence, of the corresponding fragment of the normal flbA gene in a given H.pylori strain - of the functions of the neighbouring genes.

Also included within the scope of the invention are nucleotide sequences which constitute a fragment of the flbA gene meeting the above criteria. As examples, fragments which are the subject-matter of the invention consist of at least 6 nucleotide sequences, preferably at least 50, if not at least 100 nucleotides.

Such fragments are, for example, selected either on account of their specific flbA gene character or because they belong to conserved regions of several genes encoding proteins of the LcrD/FlbF family.

According to another embodiment, the invention is also directed towards the fragments of the flbA gene which are delimited by the restriction sites which are

present in the gene. Some of these sites are defined, by way of example, in Figure 1B.

Another fragment according to the invention is a fragment of at least 1000 bp which is derived from any region of the *flbA* gene and which preferably includes a restriction site or is capable of accommodating a restriction site.

Other nucleotide sequences of the invention are, for example, recombinant nucleic acids which comprise a nucleotide sequence such as those which have been described above, itself modified by the insertion of a cassette containing a marker, for example a gene for resistance to an antibiotic or a gene for resistance to a heavy metal such as described in Application FR 9406202, which was filed on 20/05/94.

Thus, a cassette for resistance to kanamycin can be inserted. Various techniques can be used in this context and reference is made, in particular, to the paper of Labigne A. et al. (J. of Bacteriology, Vol. 170, 1988, p. 1704-1708) and the paper of Labigne A. et al. (Res. Microbiol 1992, 143, 15-26).

The invention also relates to specific oligonucleotides from a previously defined nucleotide sequence, which oligonucleotides are characterized in that they possess one of the following sequences:

OLF1bA-1: ATGCCTCGAGGTCGAAAAGCAAGATG (SEQ ID NO:1)

OLF1bA-2: GAAATCTTCATACTGGCAGCTCCAGTC (SEQ ID NO:2)

OLF1bA-7: CGGGATCCGTGGTTACTAATGGTTCTAC (SEQ ID NO:4)

OLF1bA-8: CGGGATCCTCATGGCCTCTTCAGAGACC (SEQ ID NO:5)

According to another embodiment, the invention relates to an amino acid sequence from the F1bA protein of *H.pylori*, which sequence is characterized in that it is encoded by a nucleotide sequence such as previously defined.

A specific amino acid sequence (SEQ ID NO:7) from the F1bA protein of *H.pylori* is depicted in Figure 2.

Thus, within the scope of the invention, the *flbA* gene and the protein expressed by this gene can be of interest, in particular for employment in

immunogenic compositions or compositions used for vaccination.

The invention is also directed towards bacterial strains of Helicobacter pylori which possess an aflagellate phenotype, which phenotype results from the mutation, by substitution, addition and/or deletion of bases or of a nucleotide fragment, of the above-defined nucleotide sequence of the flbA gene involved in the regulation of the biosynthesis of the flagellar proteins of H.pylori.

This modification of the flbA gene makes it possible to obtain a strain of the aflagellate type, that is which no longer expresses the FlaA and FlaB proteins and which preferably no longer expresses the proteins of the sheath.

According to one embodiment of this bacterial strain, the strain which is obtained additionally lacks the hook protein of H.pylori.

Preferably, a bacterial strain which meets the abovementioned criteria is characterized in that it is obtained from the strain N6, which was deposited in the NCIMB on 26 June 1992 under number NCIMB 40512.

By way of example, the invention relates to a recombinant aflagellate strain of H.pylori which is designated N6flbA- and was deposited in the NCIMB on 30 June 1995 under the No. NCIMB 40747.

Such aflagellate strains of H.pylori are of particular interest for employment in serology and, as a consequence, for the in-vitro detection of an infection due to H.pylori. These strains are advantageously of the recombinant type.

In particular, these strains exhibit the advantage of enabling an infection due to H.pylori to be detected in vitro in a specific and sensitive manner. In other words, the invention advantageously enables an infection due to H.pylori to be detected specifically while avoiding, in particular, "false-positive" results, for example with bacterial strains such as Salmonella or Campylobacter.

Given that the strains of H.pylori of the aflagellate type, which have thus been defined, may also have other applications, for example may be employed in the preparation of vaccine compositions, there can be interest in preparing recombinant aflagellate bacterial strains which possess a second modification or mutation, for example an aflagellate bacterial strain can be prepared which is characterized in that it is additionally mutated in such a way that it produces an attenuated urease, or even no longer produces urease, with the mutation consisting, for example, of a mutation of the nucleotide sequence of one or more genes selected from among the genes ureA, ureB, ureC, ureD, ureE, ureF, ureG, ureH or ureI. The urease structural genes, designated ureA, ureB, ureC and ureD of urease, have been described in the publication (Labigne et al (1991) J. Bacteriol. 173: 1920-1931). The other genes have been described in Patent Application EP 0610322.

The bacterial strains of the invention may be employed as such or in extract form, and, in particular, the invention relates to a total bacterial strain extract which is obtained from the previously described strains.

Such a bacterial extract can be prepared by extracting with n-octyl glucoside. In this case, the preparation technique which is employed is that described by LELWALA-GURUGE J. (Scand. J. Infect. Dis. 1992, 24: 457-465).

Another bacterial extract can be obtained by extracting with PBS or glycine using the techniques described, respectively, by BAZILLOU M. et al (Clin. Diagn. Lab. Immuno., 1994, 1: 310-317) and AGUIRRE P.M. (Eur. J. Clin. Microbiol. Infect. Dis., 1992, 11: 634-639).

Within the scope of these applications, the invention relates to a composition for the in-vitro detection of an infection due to H.pylori in a sample of biological fluid obtained from a patient, in

particular in a sample of serum, which composition includes, as the active principle, a bacterial strain of the invention or a bacterial extract in accordance with the description given above.

The biological samples which are used may be of any type and can, in particular, be any type of biological fluid, such as serum, saliva or urine, for example.

In the same way, the techniques which are employed for the detection are any techniques which involve reactions of the immunological type, in particular of the antigen/antibody type. For example, use is made of techniques such as Western blot, ELISA, etc.

The invention also relates, therefore, to a method for the in-vitro detection of an infection due to H.pylori in a sample of biological fluid taken from a patient, in particular in a sample of serum, which method comprises the steps of:

- bringing the sample under test into contact with a bacterial strain according to the invention or with a bacterial extract as defined above,

- detecting an immunological reaction between the said bacterial strain and antibodies which are directed against H.pylori and which are present in the sample under test.

By way of example, an in-vitro detection on a biological sample in order to look for an infection due to H.pylori can be carried out by implementing the following steps:

- plates are covered with the antigen which is used for the detection and which may be a pure or recombinant protein or else an aflagellate strain or a bacterial extract, in particular an NOG (n-octyl glucoside) extract of the N6flbA- strain (by way of example, the quantity of extract might be 3 µg/ml or the quantity of antigen might be 2 µg/ml),

- a range of negative and positive controls (the positive control being employed at differing dilutions) is used, and patient sera, which are diluted

to 1/100, are tested in parallel (volume deposited, 100 µl),

- an incubation step is then carried out, for example at 37°C for one hour, which step is followed by several successive washings and by a further incubation, for example at 37°C for 1 hour, with a monoclonal conjugate (of the human IgG type labelled with peroxidase), which conjugate is employed at differing dilutions (for example at a dilution of 1/32000 in the case of an antigen and at a dilution of 1/64000 in the case of a bacterial extract), with the deposited volume being 100 µl,

- after the incubation with the monoclonal conjugate, several different washings are carried out (for Example 4) and the enzymic reaction is developed, in the dark and for 30 minutes, using "OPD + substrate". The enzymic reaction is then stopped by adding H_2SO_4, after which the optical densities, OD's, are read at 492 nm/620 nm.

The invention is furthermore directed to an immunogenic composition for obtaining antibodies against H.pylori, which composition is characterized in that it includes, as the active principle, a bacterial strain according to the invention or an extract of this bacterial strain.

According to one particular embodiment of the invention, an immunogenic composition for obtaining antibodies against H.pylori is characterized in that it includes an amino acid sequence from the FlbA protein.

Also included within the scope of the present invention is a vaccinating composition for obtaining antibodies which protect against an infection due to H.pylori, characterized in that it includes, as the active principle, a bacterial strain according to the invention or a bacterial extract according to the above definitions.

Another vaccinating composition for obtaining antibodies against an infection due to H.pylori is characterized in that it includes, as the active

principle, antigens of the urease type, in particular antigens encoded by the genes ureA, ureB, ureC, or ureD and a protein having an amino acid sequence as defined above.

The invention also relates to monoclonal antibodies or polyclonal sera which are directed against a previously described amino acid sequence. These antibodies are obtained by techniques which are known per se, in particular by immunizing an animal with the chosen antigen, followed either by producing and recovering the antibodies which are produced and selecting those among them which specifically recognize H.pylori, or by preparing hybridomas, by fusing spleen cells from the previously immunized animal with myeloma cells, with these hybridomas then being cultured in order to obtain monoclonal antibodies, which are selected on the basis of the specificity with which they recognize the chosen H.pylori antigen.

Other monoclonal antibodies or polyclonal sera according to the invention are directed against an aflagellate H.pylori strain such as described in the preceding pages.

The invention furthermore relates to a composition for the in vitro detection of an infection due to H.pylori in a biological sample, which composition includes, as the active principle, monoclonal antibodies or a polyclonal serum which have been obtained against an H.pylori strain of the aflagellate phenotype according to the invention.

The invention also relates to nucleotide sequences, as the active principle of a medicament, which encode amino acid sequences according to the invention, which amino acid sequences are able to induce an immunogenic response in an animal or in a patient. A technique for employing nucleotide sequences as medicaments has been described by DONNELY et al 1995, Nature Medic. 1(6), pp. 583-587.

Figure 1

1A: Restriction map of the plasmid pILL570 and of the mini transposon Tn3 containing the cassette of the gene for resistance to kanamycin.

1B: Linear restriction maps of the recombinant plasmids pSUS39 and pSUS207. The numbers which are shown correspond to the sizes of the restriction fragments, expressed in base pairs. H: HindIII; Bg: BglII. The presence of an asterisk indicates that the restriction site was modified during the cloning and that it is no longer recognized by the corresponding restriction enzyme.

Figure 2: Nucleotide sequence (SEQ ID NO:6) of the flbA of H.pylori and the deduced amino acid sequence (SEQ ID NO:7) given in one-letter code.

Figure 3: Multiple alignment of the FlbA protein of H.pylori (SEQ ID NO:8) with five other members of the LcrD/FlbF family (SEQ ID NOS:9-13): CjFlbA: Campylobacter jejuni FlbA (SEQ ID NO:9); CcFlbF: Caulobacter crescentus FlbF (SEQ ID NO:10); YpLcrD: Yersinia pestis LcrD (SEQ ID NO:11); StInvA: Salmonella typhimurium InvA (SEQ ID NO:12); SfMxiA: Shigella flexneri MxiA (SEQ ID NO:13). The asterisks indicate the positions of the amino acids which are conserved in all the homologs of the LcrD/FlbF family; the dots indicate the positions of the amino acids which are conserved in at least 5 out of the 6 homologous proteins; the conserved amino acid sequences which were used for synthesizing the degenerate oligonucleotides (OLFlbA-1 and OLFlbA-2) are underlined. Particular note should be taken of the degree of conservation of the N-terminal domain of these homologous proteins, which contrasts with the degree of variability of the hydrophilic domain of the C-terminal region.

Figure 4: Diagrammatic depiction of the phylogenetic tree of six proteins belonging to the LcrD/FlbF family. The proteins which are involved in regulating the expression of mobility, i.e. FlbA of H.pylori (HpFlbA) and of Campylobacter jejuni (CjFlbA), and FlbF of Caulobacter crescentus (CcFlbF) form a branch which is distinct from that of the proteins involved in the

secretion of virulence proteins (InvA, MxiA and LcrD of Salmonella, Shigella and Yersinia, respectively). The numbers which are shown depict the relative evolutionary distance.

Figure 5: Diagrammatic representation of the strategy which was followed for constructing the isogenic mutants of H.pylori strain N6, i.e. mutants in which the gene encoding the FlbA protein was inactivated by inserting a gene encoding for resistance to kanamycin.

Figure 6: Analysis by immunoblotting (Western blot) of the proteins from an N6-flbA⁻ mutant using AK179 antiserum (3), which is specifically directed against flagella which have been purified from H.pylori: 1: N6-flbA mutant; 2: flaA/flaB double mutant; 3: flaB (8) mutant; 4: flaA (8) mutant; 5: wild-type N6 strain.

Figures 7 to 11: Comparative results from the serology carried out on H.pylori.

Figures 12 and 13: Extractions using the aflagellate strain N6flbA-: the extractions were carried out using glycine, PBS or NOG.

Figure 12: The curves were constructed on the basis of the following data:

STD#	CONC	NET ABS 750.0	CALC CONC	DIFF	COEFFS:
1	0.0000	0.0020	-0.003	0.0080	P2=2.0324
2	0.1660	0.0760	0.1721	-0.006	P1=2.2753
3	0.3300	0.1400	0.3459	-0.016	P0=0
4	0.6650	0.2390	0.6474	0.0176	
5	1.3300	0.4280	1.3336	-0.004	

MEAN:
-1.0356E-07
S.D.: 0.0130

Figure 13: Minimethod (BIO-RAD) protein assays
Glycine: diluted 1/2; glucoside: diluted 1/10; supernatant 1: diluted 1/4; supernatant 2: not diluted.

The curves were constructed on the basis of the following data:

STD#	CONC	NET ABS 750.0	CALC CONC	DIFF	COEFFS:
1	0.0000	-0.003	1.5398	-1.540	P2=144.63
2	25.000	0.0600	21.861	3.1392	P1=314.31
3	50.000	0.1470	51.810	-1.810	P0=2.4815
4	100.00	0.2750	99.855	0.1454	
5	200.00	0.5090	199.94	0.0636	

EXAMPLES

I Identification of the flbA gene and preparation of aflagellate strains

Among the proteins which are known to play a role in regulating the expression of bacterial mobility, the proteins belonging to the recently identified LcrD/FlbF family, which include the LcrD protein of the bacteria of the genus *Yersinia* (6), the InvA protein of *Salmonella* (2), MxiA of *Shigella* (1), FlbF of *Caulobacter crescentus* (7) and LfbA of *Campylobacter jejuni* (4), are proteins of interest. The LcrD , InvA and Mxia proteins are involved in the regulation and/or the secretion of proteins which are associated with the virulence of the bacteria which express them, whereas the FlbF protein of *Caulobacter crescentus* and the FlbA protein of *Campylobacter jejuni* are involved in regulating the biosynthesis of the flagella and therefore involved in regulating mobility. The homologs of the LcrD/FlbA family which are known to date possess very conserved domains, especially in the N-terminal part of these proteins, and it was therefore possible to use two of these conserved regions (MPGKQM, amino acids 151 to 156 of the LcrD protein of *Yersinia*) and MDGAMKF (amino acids 189 to 195 of LcrD) for defining two degenerate oligonucleotides (OLFlbA-1 and OLFlbA-2, Table 1), which were synthesized and which have served as nucleotide primers in the gene amplification experiments which were carried out on the chromosomal DNA of *Helicobacter pylori*. In this way, it was possible to amplify a fragment of 130 base pairs (bp), and determination of its nucleotide sequence

demonstrated that this fragment encoded a segment of a protein which was very homologous to the proteins of the LcrD/FlbF family. This amplified fragment was then labelled radioactively and used as a probe to screen an H.pylori cosmid library.

This fragment corresponds to the sequence contained between nucleotides 575 and 707 of the sequence depicted in Figure 2. (SEQ ID NO:6)

One of the cosmids of the genomic library was identified as encoding the LcrD/FlbF homolog of H.pylori and was then subjected to a partial digestion with Sau3A so as to construct a mini library (200 subclones) of the cosmid in vector pILL570, containing inserted fragments possessing a size of between 2 and 5 (kilobases). Vector pILL570 has been described in the paper by Labigne A. et al (Institut Pasteur/Elsevier Paris 1992. Res. Microbiol. 1992, 143, 15-26). Its restriction map is given in Figure 1A. These 200 clones were then hybridized to the 130 bp probe, and the clones which harboured plasmids pSUS39 and pSUS207 gave a positive hybridization. The linear restriction maps of these two recombinant plasmids are depicted in Figure 1B and demonstrate that the two inserts of these clones have overlapping sequences. Determination of the nucleotide sequences of these two inserts revealed that neither of the two inserts contained the flbA gene in its entirety. The flbA gene of H.pylori, designated in this way due to its homology with the flbA gene of Campylobacter jejuni, corresponds to an open reading frame of 2196 nucleotides and encodes a protein having a calculated molecular mass of 80.1 kilodaltons. The nucleotide sequence of flbA (SEQ ID NO:6) and the amino acid sequence (SEQ ID NO:7) of FlbA are given in Figure 2. Consensus sequences which are characteristic for promoter or terminator sequences have not been detected upstream and downstream of the open reading frame.

The FlbA protein exhibits similarities with the FlbA protein of Campylobacter jejuni and the FlbF protein of Caulobacter crescentus, both of which are

involved in mobility (51.7% and 40.4% identity, respectively) whereas these percentages are lower with members of the LcrD/FlbF protein family which are not involved in mobility: 32.8% identity with LcrD from Yersinia, 30.5% with MxiA from Shigella and 29.3% with InvA from Salmonella. A multiple alignment of the amino acid sequences of these proteins (SEQ ID NOS:9-13) with that of H.pylori FlbA (SEQ ID NO:8) is given in Figure 3. The most conserved regions of the homologs of the LcrD/FlbF family are located in the N-terminal part of the proteins.

The phylogenetic evolution of the proteins involved in mobility (FlbA and FlbF) and that of the proteins involved in regulating the expression and/or the secretion of proteins associated with virulence is depicted diagrammatically by a phylogenetic tree (Figure 4). Two distinct branches can be seen; H.pylori FlbA belongs unambiguously to the branch corresponding to the regulatory proteins involved in the biosynthesis of the flagella.

Construction and characterization of isogenic mutants of H.pylori which are deficient in the synthesis of the FlbA protein.

A 1600 base pair fragment was amplified from plasmid pSUS39 using the oligonucleotides OLFlbA-7 and OLFlbA-8 (Table 1), each of which contains a BamHI restriction site at its 5' end. In its central region, this amplified fragment contains a unique HindIII restriction endonuclease site and was cloned into vector pSUS33, which is a derivative of plasmid pUC19 in which the HindIII site situated in the multiple cloning site has been deleted. In order to obtain pSUS33, plasmid pUC19 was restricted with HindIII; the sticky ends resulting from this restriction were treated with Klenow enzyme and T4 DNA polymerase in order to produce blunt ends; the resulting fragment was religated with T4 DNA ligase and introduced into E.coli DH5x in order to produce pSUS33. The recombinant plasmid resulting from the integration of the 1600 base pair fragment into pSUS33 was designated pSUS40; it was

linearized with HindIII, its ends were blunt-ended and the SmaI kanamycin cassette, which was derived from plasmid pILL600 (Labigne A. et al, 1988, J. Bact. 170, 1704-1708), was cloned into this unique site, resulting in plasmid pSUS42. Plasmid pSUS42 was then introduced by electroporation into the "N6" strain of H.pylori. The electroporation was carried out in accordance with the technique described by Ferrero R.L. et al (Journal of Bacteriology, July 1992, pp. 4212-4217, Vol. 174, No. 13). The transformants which were obtained after selecting on a selective medium containing kanamycin (25 µg/ml) were then characterized genotypically and phenotypically. Figure 5 shows a diagram of the procedure which was followed for the construction of mutants. Genotypic characterization of these mutants, by gene amplification and Southern hybridization, demonstrated that the genomes of the transformants which were resistant to kanamycin contained the resistance gene inserted in the middle of the flbA gene and that there had therefore been an allelic replacement, by means of double crossing-over, of the wild-type copy of the flbA gene by the inactive flbA-Km copy, with the loss of the nucleotide sequences of the pSUS33 vector. Phenotypic characterization of the flbA⁻ mutants of H.pylori demonstrated that they were not mobile; furthermore, analysis of these mutants by electron microscopy revealed that there was a total absence of the flagellum elements and an absence of the flagellum sheath. The immunoblotting experiments (Western blots) which were carried out using antibodies directed against the proteins of the entire flagellum of H.pylori (Figure 6) demonstrated that two peptide bands corresponding to the flagellar subunits FlaA and FlaB were absent, as was a band corresponding to a polypeptide of an apparent mass of 90 kilodaltons, which is a protein which has recently been identified by O'Toole and collaborators (5) as being the hook protein (or anchoring protein) of the flagellum (5).

Taken as a whole, these results suggest that the FlbA protein of H.pylori is essential for the biosynthesis of all the flagellar structures and that inactivation of the gene encoding this protein results in complete cessation of the synthesis of any structure entering into the formation of the flagellum and not in interruption of the export of the constituents of these structures.

SUB F3

Table 1: Oligonucleotides employed in this study

Oligonucleotide	Position	Strand	Nucleotide sequence
OLF1bA-1	AS 151-156 (LcrD)	+	ATGCCTCGAGGTCGAAAAGCAACATG (SEQ ID NO:1)
OLF1bA-2	AS 189-195 (LcrD)	-	GAAATCTTCATACTGGCAGCTCCASTC (SEQ ID NO:2)
OLF1bA-7	515-534	+	CGGGATCCGTGGTTACTAATGGTTCTAC (SEQ ID NO:4)
OLF1bA-8	2092-2111	-	CGGGATCCTCATGGCCTCTTCAGAGACC (SEQ ID NO:5)

II H.pylori serology

- Models studied

1) HspAmalE — recombinant protein of 47.5 kD (HspA=13 kD)
A sensitivity of 41% and a specificity of 96% were obtained on the population termed population 1 of documented sera.

2) N6flbA- — aflagellate strain of Helicobacter pylori
3 extractions were carried out:
- n-Octyl glucoside
- PBS
- Glycine
For the time being, the extraction with n-octyl glucoside (NOG) appears to be the best.

3) -N6 — corresponding wild-type strain

An extraction was carried out with n-octyl glucoside.

A second population of sera was employed (population II). This population consists of some one hundred sera which are well documented from the clinical, endoscopic, histological, bacteriological and anatomopathological points of view. It was this population II which was used to assess the performances of the different models under study. Five different populations were tested.

- 5 populations of tested sera:
  - 300 ordinary sera (ENTS)
  - 18 sera which were positive by WHITTAKER serology (CBMS)
  - 92 well documented sera termed sera of population II
  - 87 sera which were documented from the bacteriological and anatomopathological points of view and which were termed sera of population I.
  - 23 sera exhibiting cross reactions:
    - 10 anti-Legionella positive sera
    - 10 anti-Chlamydia positive sera
    - 3 anti-Campylobacter positive sera

Two competing kits, which bibliographic studies indicated were effective, were tested in parallel.

- 2 tested commercial kits:
  - Cobas Core (ROCHE)
  - Pylori Stat (WHITTAKER)

- Results

The ordinary sera (ENTS) (Figures 8 to 11, Table 2)

- 300 sera were taken through the following models:
  - Hsp A malE
  - N6 flBA-
  - N6

The epidemiological studies give seroprevalences, in France, of between 20 and 25%. The distribution of 300 blood donor sera was studied and the prevalence of positivity was calculated for different threshold values in order to validate the threshold value which was previously defined using the CBMS serum library (WHITTAKER serology).

This study enables the different tests to be compared using the same seroprevalence.

- The first 43 sera were also taken through the following models:
  - Cobas Core (ROCHE)
  - Pylori Stat (WHITTAKER)
  - serology known as JLF serology (ELISA test, based on an aqueous extract of several bacterial strains)

The results are expressed in arbitrary units and for different threshold values; a positive result is written as 1 and a negative result is written as 0.

On comparing these 43 sera in different tests, it can be observed that:

- the aflagellate strain N5flbA- and the Cobas Core test (Roche) give comparable seroprevalences of the order of 20%.
- HspA gives a very low seroprevalence (7%), which suggests a lack of sensitivity in view of the subsequent results.
- the JLF serology appears to be very specific since the seroprevalence is only 14%, considering the subsequent results.
- the Pylori Stat test (Whittaker) gives a high seroprevalence (29%), which might indicate a lack of specificity or a threshold value which is too low.

Table 2A Comparison of 43 FNTS sera with regard to:

No.	HBsA	150	C50+H	C30+H	C.Con	P	P. Sat	0.36	NBHBA- PBS	100	80	60	NBB	100	80	60	JLP Sero.	0.30	NB NBB	100
1	0	0	1	1	170	1	0.70	1	3393	1	1	1	3105	1	1	1	0.75	1	>928	1
2	0	0	0	0	0	0	0.21	0	4	0	0	0	3	0	0	0	0.01	0	5	0
3	0	0	0	0	3	0	0.23	0	0	0	0	0	4	0	0	0	0.01	0	0	0
4	0	0	0	0	4	0	0.18	0	4	0	0	0	3	0	0	0	0.02	0	4	0
5	0	0	0	0	3	0	0.20	0	12	0	0	0	0	0	0	0	0.08	0	27	0
6	1	0	0	0	2	0	0.17	0	0	0	0	0	0	0	0	0	0.01	0	0	0
7	2	0	0	0	3	0	0.32	0	41	0	0	0	33	0	0	0	0.02	0	13	0
8	78	0	0	0	137	1	0.57	1	3391	1	1	1	3109	1	1	1	1.43	1	>928	1
9	0	0	0	0	3	0	0.35	1	23	0	0	0	21	0	0	0	0.06	0	18	0
10	0	0	0	0	1	0	0.34	0	28	0	0	0	14	0	0	0	0.02	0	31	0
11	0	0	0	0	3	0	0.23	0	61	0	0	1	33	0	0	0	0.00	0	77	0
12	0	0	0	0	0	0	0.18	0	0	0	0	0	0	0	0	0	0.02	0	0	0
13	0	0	0	0	1	0	0.19	0	51	0	0	0	28	0	0	0	0.02	0	57	0
14	0	0	0	0	3	0	0.25	0	4	0	0	0	4	0	0	0	0.02	0	11	0
15	1	0	0	0	3	0	0.27	0	4	0	0	0	3	0	0	0	0.02	0	7	0
16	0	0	0	0	0	0	0.37	1	82	0	0	1	31	0	0	0	0.05	0	87	0
17	0	0	0	0	1	0	0.22	0	5	0	0	0	2	0	0	0	0.03	0	13	0
18	0	0	0	0	1	0	0.23	0	13	0	0	0	8	0	0	0	0.00	0	18	0
19	0	0	0	0	0	0	0.27	0	4	0	0	0	4	0	0	0	0.08	0	15	0
20	0	0	0	0	1	0	0.21	0	23	0	0	0	12	0	0	0	0.01	0	31	0
21	0	0	0	0	18	1	0.40	1	54	0	0	0	204	1	1	1	0.24	0	331	1

Table 2B Comparison of 43 FNTG wera with regard to:

No.	HipA	150	C50+H	C30+H	C.Cons	S	P. Stat	0.35	MBB	100	80	60	NOO	100	80	60	ULP Sero.	0.30	NS NOO	NS 100
22		0	0	0	1	0	0.43	1	13	0	0	0	40	0	0	0	0.05	0	21	0
23	0	0	0	1	25	1	0.49	1	265	1	1	1	298	1	1	1	0.37	1	>928	1
24	9	0	1	1	125	1	0.65	1	3300	1	1	1	3100	1	1	1	1.47	1	>928	1
25	0	0	3	0	0	0	0.20	0	34	0	0	0	20	0	0	0	0.01	0	51	0
26	0	0	0	0	2	0	0.28	0	97	0	1	1	60	0	0	0	0.04	0	105	1
27	0	0	1	1	10	1	0.33	0	265	1	1	1	339	1	1	1	0.07	0	359	1
28	0	0	1	1	7	0	0.21	0	28	0	0	0	14	0	0	0	0.05	0	27	0
29	3	0	0	0	8	0	0.20	0	3	0	0	0	2	0	0	0	0.01	0	0	0
30	1	0	0	0	2	0	0.23	0	3	0	0	0	3	0	0	0	0.01	0	4	0
31	0	0	0	0	1	0	0.23	0	0	0	0	0	1	0	0	0	0.03	0	0	0
32	0	0	0	0	2	0	0.31	0	24	0	0	0	15	0	0	0	0.05	0	30	0
33	0	0	0	0	1	0	0.23	0	14	0	0	0	8	0	0	0	0.02	0	8	0
34	0	0	0	0	3	0	0.23	0	0	0	0	0	1	0	0	0	0.00	0	2	0
35	1203	1	1	1	170	1	0.84	1	463	1	1	1	3103	1	1	1	0.00	1	>928	1
36	0	0	0	0	4	0	0.36	1	42	0	0	0	32	0	0	0	0.04	0	60	0
37	0	0	0	0	5	0	0.22	0	110	1	1	1	100	1	1	1	0.02	0	227	1
38	13	0	0	0	4	0	0.47	1	17	0	0	1	60	0	0	1	0.07	0	108	1
39	1	0	0	0	4	0	0.34	0	23	0	0	0	13	0	0	0	0.05	0	34	0
40	0	0	0	0	2	0	0.19	0	5	0	0	0	8	0	0	0	0.02	0	4	0
41	0	0	0	0	0	0	0.24	0	48	0	0	0	23	0	0	0	0.05	0	88	0
42	0	0	0	0	170	1	0.59	1	3380	1	1	1	3104	1	1	1	1.47	1	>928	1
43	0	0	1	1	3	0	0.19	0	7	0	0	0	3	0	0	0	0.04	0	8	0
	No. of 1's	1 2%	8 14%	7 16%		8 19%		12 28%		8 19%	9 21%	12 28%		9 21%	9 21%	10 23%		6 14%		11 26%

<u>The sera which are positive by WHITTAKER serology (CBMS) (Table 3)</u>

Three sera were found to be positive only with the Pylori Stat test (Whittaker). They were not confirmed using any other test.

It may be supposed that this result is due to this test lacking specificity. If the Cobas Core test (Roche), which is one of the best which is currently on the market, is taken as the reference, we can compare our different models in relation to Cobas Core.

- The aflagellate N6flbA- strain correlates perfectly with Cobas Core.

- The 3 sera which are negative with Cobas Core are also negative with N6flbA-

- The 15 sera which are positive with Cobas Core are also positive with N6flbA-.

- The wild-type N6 strain gives the same results as the aflagellate strain.

- HspA also lacks sensitivity since 9 Cobas Core-positive sera are negative with HspA.

The 3 sera which are negative with Cobas Core are also negative with HspA.

Table 3

19 CBMS sera which are positive by WHITTAKER serology (Pylori Stat)

No. of Serum	OD	HspA	160	C. Core	8	NGMBA PBS	80	NOG	60	GLY	N6 NOG	100
1	1.8	0		33	1	130	1	289	1	494	830	1.
2	2.41	607	1	>80	1	471	1	3257	1	6587	>928	1
3	2.9	675	1	30	1	472	1	3263	1	1183	>928	1
4	1.4	146		42	1	158	1	407	1	825	556	1
5	1	179	1	44	1	59	0	81	1	317	276	1
6	2.6	183	1	>80	1	.472	1	3260	1	1054	>928	1
A	0.7	19		4		13		8		33	12	
B	2.6	5		>60	1	471	1	3255	1	6600	>928	1
C	3.1	1352	1	>80	1	470	1	3246	1	6582	>928	1
D	1.3	3		18	1	121	1	506	1	446	>928	1
E	0.6	7		1		23		45		150	0	
F	2.1	0		15	1	.139	1	3258	1	280	>928	1
G	0.2	0		8		3		4		28	0	
H	1.4	25		19	1	127	1	178	1	143	159	1
I	2.3	960	1	>80	1							
J	1.9	5		38	1	91	1	117	1	57	101	1
K	1.38	4		52	1	88	1	182	1	167	>928	1
L	2.98	855	1	>80	1	471	1	586	1	943	>928	1
M	2.86	0		51	1	471	1	3256	1	1200	>928	1

The sera of population II

92 sera were selected, with the sera dividing into 3 groups:

-34: dyspeptic patients
diagnosis of ulcer (duodenal or gastric) by endoscopy and histology
presence of Helicobacter pylori by culture and/or anatamopathologically; a rapid urea test was also carried out.
This group will be termed Hp+/U+

-27: dyspeptic patients
differential diagnosis of ulcer (gastritis etc.) by endoscopy and histology
presence of Helicobacter pylori by culture and/or anatamopathologically; a rapid urea test was also carried out.
This group will be termed Hp+/U-

-31: patients which are or are not dyspeptic
normal gastroduodenum by endoscopy and histology
absence of Helicobacter pylori by culture and anamatopathologically; a rapid urea test was also carried out.
This group will be termed Hp-

The clinical, endoscopic, histological, bacteriological and anatomopathological findings are indicated for each patient.
This well documented population enabled criteria of sensitivity and specificity to be defined.

- HpA: A substantial lack of sensitivity, as observed with population I, is still noticed.
The sensitivity is 59%, with a specificity of 100.

-N6flbA: A sensitivity of 100% is confirmed for the n-octyl glucoside extract, with a specificity of 90%.
This result is comparable to that obtained with the Roche Cobas Core test (98% sensitivity with a specificity of 94%).

-N6: On population II, the wild-type strain is entirely comparable to the aflagellate strain.
None of the 31 negative sera is positive with the wild-type strain; no cross reaction due to the flagellum was detected with this population II.

Table 4: Sera of population II

34 Hp+/U+ patients

No.	Date	Age	Sex	clinical	Endo	UU/UD	HLA	Giemsa	Cult	U??	HP
9	18/07/91	33	1	dyspepsia	DU	1	0710	0	1	0	1
11	17/03/92	37	1	dyspepsia	DU	1	0313	1	1	1	1
13	10/03/92	18	1	dyspepsia	DU	1	0311	1	0	0	1
15	23/01/93	71	1	dyspepsia	DU	1	0222	1	1	1	1
16	10/12/91	37	1	dyspepsia	DU	1	0410	1	0	0	1
17	18/01/94	40	1	perforation	DU	1	0230	1	0	1	1
18	16/12/92	22	1	dyspepsia	DU	1	0121	1	0	1	1
19	07/09.94	39	1	dyspepsia	DU	1	0120	1	1	1	1
21	05/07/92	47	0	dyspepsia	DU	1	0212	1	1	1	1
28	27/04/94	41	1	CON-A, GU	GU/DU	1	0320	1	1	1	1
29	24/09/94	37	1	A[illegible] dysphagia	CON-U	1	0722	0	1	0	1
33	04/11/91	60	1	dyspepsia	DU	1	0231	1	1	1	1
38	03/08/90	46	1	dyspepsia	GU	1	0331F	1	0	0	1
39	07/03/94	18	1	APC dyspepsia	GU	1	0212	1	1	1	1
43	13/01/94	67	0	APC dyspepsia	DU	1	0111	1	0	1	1
44	01/01/??	60	0	dyspepsia	DU	1	0321	1	1	1	1

No.	HpX	VI=150	FBI	VI=10	KOO	VS=60	KOO	VS=10	C.COK	VL=1	PJYAY	VS=0.11/0.31	JLP	VS=0.50
9	8		180	1	303	1	>928	1	30	1	0.188	1	0.81	1
11	51		>484	1	1000	1	>928	1	58	1	0.359	1	1.03	1
13	1530	1	>484	1	1452	1	>928	1	>180	1	0.377	1	0.61	1
15	42		131	1	278	1	409	1	40	1	0.723	1	0.78	0
16	2130	1	>484	1	870	1	>928	1	80	1	0.300	1	1.14	1
17	14		>484	1	878	1	>928	1	87	1	0.302	1	1.17	1
18	10		>484	1	1174	1	>928	1	>180	1	0.313	1	0.74	1
19	11		69	1	64	1	409	1	10	1	0.709	1	0.74	1
21	12		58		104	1	398	1	18	1	0.745	1	0.73	0
28	1110	1	>484	1	2344	1	>928	1	>180	1	0.477	1	1.73	1
29	803	1	>484	1	2380	1	>928	1	>180	1	0.483	1	1.51	1
33	883	1	>484	1	7720	1	>928	1	>180	1	0.505	1	0.97	1
38	4660	1	>484	1	2876	1	>928	1	>180	1	0.419	1	1.34	1
39	772	1	149	1	438	1	>928	1	14	1	0.733	1	0.88	1
43	108		18		15	1	319	1	14	1	0.704	1	0.57	1
44	9		>484	1	2878	1	>928	1	>180	1	0.485	1	1.09	1

Table 4 (continued): Sera of population II

34 Hp+/U+ patients

No.	Date	Age	Sex	Clinical	Endos.	UO/UO	HLA	Dikmen	Cvd	Ure	HP
48	02/02/93	60	1	dyspepsia	UD	1	0120	1	1	0	1
52	18/10/94	45	1	dyspepsia AU	GDN	1	0721F	1	1	1	1
53	05/03/94	72	0	dyspepsia	UD	1	0733	1	0	1	1
60	12/09/91	40	1	dyspepsia	UO	1	0333	0	1	0	1
64	01/11/94	31	1	ort. dyspepsia	UD	1	0323	1	1	1	1
65	15/12/94	41	1	perforation	UD	1	0111F	1	1	1	1
70	22/01/93	47	1	dyspepsia-melaena	UD	1	0113	1	0	0	1
71	18/03/93	38	1	dyspepsia	UD	1	2221	1	0	1	1
75	09/08/93	38	1	dyspepsia	UO	1	0121*	1	0	0	1
78	12/05/93	19	0	dyspepsia AU	UO	1	0720	1	0	1	1
81	08/08/94	29	1	dyspepsia, A DU	OON	1	0110	1	1	1	1
84	22/11/93	24	1	perforation	GDN	1	0773	1	0	1	1
88	17/06/93	38	1	dyspepsia	UD	1	0111	1	0	0	1
89	22/06/94	23	1	dyspepsia	UD	1	0772	1	1	1	1
90	23/05/94	41	1	dyspepsia	UD	1	0331	1	1	1	1
91	01/06/93	57	1	ch dyspepsia-x	UD	1	0123	1	1	1	1
99	15/07/97	34	0	dyspepsia	UD	1	0321	1	0	1	1
100	13/06/97	31	1	dyspepsia	UD	1	0221	0	1	1	1

No.	HpA	VIII(150)	P84	V3=60	HOO	V3=20	HOO (R6)	V3=15	CZOK	V3=8	P.BYAT	V3=0.11/0.37	JLY	V3=0.30
48	39	[illegible]	173	1	304	1	>973	1	100	1	0.774	1	1.50	1
52	6	[illegible]	154	1	308	1	505	1	18	1	0.757	1	0.77	0
53	1670	1	>464	1	1704	1	>978	1	85	1	0.368	1	1.21	1
60	3400	1	>464	1	3197	1	>978	1	>100	1	0.324	1	0.18	1
64	7	[illegible]	173	1	870	1	>924	1	40	1	0.367	1	0.39	1
65	3	[illegible]	100	1	310	1	>978	1	86	1	0.318	1	0.33	1
70	6	[illegible]	164	1	384	1	804	1	23	1	0.798	1	1.13	1
71	107	[illegible]	97	1	121	1	709	1	73	1	0.183	[illegible]	0.14	0
75	313	1	140	1	720	1	785	1	37	1	0.798	1	0.73	0
78	1333	1	>464	1	930	1	>978	1	>100	1	0.394	1	1.18	1
81	111	[illegible]	90	1	173	1	>978	1	30	1	0.702	1	0.77	1
84	74	[illegible]	>464	1	842	1	>978	1	68	1	0.316	1	0.84	1
88	150	1	>464	1	440	1	>978	1	43	1	0.185	1	3.80	1
89	18	[illegible]	175	1	712	1	>975	1	117	1	0.344	1	0.51	1
90	80	[illegible]	384	1	812	1	>978	1	91	1	0.349	1	0.78	1
91	11	[illegible]	730	1	177	1	175	1	61	1	0.188	[illegible]	0.17	0
99	21	[illegible]	294	1	310	1	>978	1	79	1	0.362	1	1.58	1
100	70	[illegible]	415	1	444	1	359	1	87	1	0.388	1	0.31	1

Aflagellate variant verita

Table 5: Sera of population II

27 Hp+/U+ patients

N°	Date	Age	Sex	Clinical	Endos.	UG/UD	Pyl.	Giemsa	HP cul	Ure	HP	HpA	VE=150	Allogeneic ... titres: F83	VE=10	NOO	VE=64	HI NOO	VS=14	C.COR	VB=1	P.STAT	VB=0.15-0.3	ZF	VB=0.30
1	11/04/93	24	0	urt. dyspepsia	GDN	0	0721	1	1	1	1	219	1	>464	1	661	1	>978	1	42	1	0.183	1	0.43	1
2	26/05/94	45	1	An. dyspepsia	ODN	0	G311	1	1	0	1	33	[illegible]	>464	1	1690	1	>978	1	>160	1	0.311	1	0.50	1
3	08/04/92	44	1	dyspepsia	GDN	0	0313	1	1	1	1	63	[illegible]	384	1	400	1	480	1	22	1	0.198	1	0.05	0
4	01/12/94	28	0	dyspepsia	ODN	0	G321F	1	1	0	1	78	[illegible]	>464	1	1760	1	>978	1	40	1	0.781	1	0.58	1
6	22/08/94	28	0	dyspepsia	ODN	0	G220	0	1	0	1	14	[illegible]	704	1	354	1	374	1	18	1	0.333	1	0.18	0
7	21/01/93	58	0	urt. dyspepsia	GDN	0	G320	1	1	1	1	710	1	>464	1	2058	1	>920	1	77	1	0.373	1	0.41	1
12	04/11/92	48	0	Dyspepsia-GUR	GCN	0	G310	1	1	1	1	341	1	251	1	805	1	>978	1	101	1	0.343	1	0.11	1
13	01/12/94	28	0	dyspepsia	GCN	0	G312	1	1	0	1	1550	1	460	1	452	1	>978	1	47	1	0.33	1	0.85	1
14	21/04/93	39	1	urt. GUR	ODN	0	0321	1	0	1	1	30	[illegible]	84	1	149	1	>978	1	37	1	0.737	1	0.15	0
15	09/11.94	76	0	An. dyspepsia	OCN	0	0121	1	1	0	1	3750	1	>464	1	1384	1	>978	1	>100	1	0.41	1	0.15	1
31	21/01/93	53	1	dyspepsia	GDN	0	0333	1	0	0	1	3870	1	>464	1	3400	1	>978	1	>100	1	0.493	1	3.89	1
32	08/11/93	53	0	alt. dyspepsia	OCN	0	0211	1	0	1	1	81	[illegible]	453	1	850	1	>978	1	45	1	0.336	1	0.35	1
37	01/12/93	50	1	dyspepsia	Erosions G	0	G111	1	0	0	1	71	[illegible]	345	1	456	1	>978	1	77	1	0.3	1	0.87	1
42	16/12/94	20	1	dyspepsia	GCN	0	0321F	0	1	0	1	423	1	>464	1	1007	1	>978	1	124	1	0.404	1	0.61	1
45	08/06/94	46	0	urt. dyspepsia	ODN	0	0310	1	1	1	1	747	1	222	1	431	1	>978	1	104	1	0.325	1	1.07	1
49	17/05/93	43	1	urt. dyspepsia	OCN	0	0210	1	0	1	1	37	[illegible]	47	[illegible]	61	1	251	1	38	1	0.179	[illegible]	0.28	0
55	21/06/93	46	0	anaemic(?) dyspepsia	ODN	0	0333	1	0	1	1	2225	1	>464	1	785	1	>978	1	100	1	0.374	1	0.47	1
58	19/12/94	58	0	An. dyspepsia	GDN	0	0310	1	0	0	1	1615	1	>404	1	187	1	>978	1	61	1	0.323	1	0.83	1
59	08/06/93	30	0	GUR	ODN	0	0111	1	0	1	1	0	[illegible]	80	[illegible]	134	1	234	1	8	[illegible]	0.180	[illegible]	0.44	1
53	06/01/93	55	1	dyspepsia	ODN	0	G222	0	1	0	1	72	[illegible]	>464	1	1008	1	>978	1	88	1	0.332	1	0.48	1
51	01/06/94	31	0	vomiting	GDN	0	G322	0	1	1	1	72	[illegible]	134	1	184	1	188	1	17	1	0.203	1	0.73	0
61	23/08/93	79	0	urt. dyspepsia	ODN	0	0722	1	0	1	1	115	1	>464	1	401	1	406	1	36	1	0.278	1	0.33	1
73	27/06/94	60	1	dyspepsia	GDN	0	0712	1	1	1	1	812	1	158	1	309	1	>978	1	148	1	0.317	1	1.28	1
74	20/04/94	62	0	dyspepsia	ODN	0	0722	1	1	1	1	4850	1	310	1	2156	1	>978	1	>160	1	0.372	1	1.01	1
77	04/11/92	71	1	anaemic(?) dyspepsia	GDN	0	0211	1	0	0	1	13	[illegible]	142	1	210	1	400	1	70	1	0.238	1	0.79	0
85	21/11/94	51	1	GUR	GDN	0	G111F	1	1	1	1	2	[illegible]	187	1	324	1	170	1	28	1	0.247	1	0.18	0
93	07/12/94	42	0	GUR	ODN	0	0321	1	1	1	1	59	[illegible]	179	1	357	1	>978	1	173	1	0.781	1	1.54	1

Table 5a: Sera of population II

31 Hp- patients

No	Dt	Age	Sex	Clinics	Endos.	UDP00	Histo	Hp Giemsa	Hp Cul	Hp Ure	H?	IgA	VS=131	Flagellate FAS	VS=10	HOO	VS=10	X6 HOA	VS=100	C.COR	VI=	F.STAT	VS=0.1(0.2)	JCP	VS=0.30
8	19/01/92	44	1	CDR/urt. dyspepsia	GDN	0	normal	0	0	0	0	73	0	34	0	12	0	11	0	8	1	0.158	0	0.01	0
14	07/08/93	34	1	urt. dyspepsia	ODN	0	normal	0	0	0	0	8	0	0	0	0	0	0	0	3	0	0.1	0	0.00	0
19	11/02/93	43	1	dyspepsia	GDN	0	normal	0	0	0	0	18	0	15	0	3	0	31	0	8	0	0.173	0	0.13	0
20	23/11/84	72	1	corticoide	ODN	0	normal	0	0	0	0	7	0	0	0	0	0	0	0	3	0	0.166	0	0.01	0
21	15/03/93	85	1	dyspepsia	ODN	0	normal	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0.177	0	0.06	0
22	18/11/84	33	1	urt. dyspepsia	ODN	0	normal	0	0	0	0	2	0	64	0	40	0	68	0	0	0	0.152	0	0.04	0
30	25/01/85	39	0	dyspepsia	GDN	0	normal	0	0	0	0	10	0	28	0	12	0	24	0	4	0	0.000	0	0.00	0
34	13/01/84	74	1	dyspepsia	ODN	0	normal	0	0	0	0	29	0	36	0	15	0	56	0	6	0	0.170	0	0.00	0
39	14/11/84	84	0	[illegible]	OON	0	normal	0	0	0	0	28	0	35	0	14	0	82	0	3	0	0.135	0	0.00	0
38	31/01/84	43	1	dyspepsia	ODN	0	normal	0	0	0	0	9	0	4	0	2	0	22	0	4	0	0.113	0	0.08	3
41	21/04/93	37	1	urt. dyspepsia	GCN	0	normal	0	0	0	0	15	0	172	[illegible]	108	[illegible]	219	[illegible]	9	[illegible]	0.17	0	0.00	0
46	07/10/92	38	1	dyspepsia	GCN	0	normal	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0.133	0	0.00	0
47	21/01/93	40	0	urt. dyspepsia	GCN	0	normal	0	0	0	0	7	0	114	[illegible]	90	[illegible]	199	[illegible]	8	0	0.153	0	0.00	0
50	08/07/82	19	1	dyspepsia	ODN	0	normal	0	0	0	0	4	0	11	0	5	0	13	0	0	0	0.13	0	0.01	0
51	10/02/83	28	1	urt. dyspepsia	OCN	0	normal	0	0	0	0	14	0	7	0	2	0	7	0	4	0	0.119	0	0.00	3
54	24/07/83	51	1	GCA	ODN	0	normal	0	0	0	0	8	0	24	0	11	0	29	0	3	0	0.143	0	0.00	3
61	06/02/91	60	1	GUR	ODN	0	normal	0	0	0	0	33	0	61	0	80	[illegible]	436	[illegible]	8	0	0.777	1	0.38	1
61	11/01/86	42	1	dyspepsia	GDN	0	normal	0	0	0	0	9	0	10	0	2	0	11	0	0	0	0.082	0	0.00	0
67	21/07/93	37	0	dyspepsia	ODN	0	normal	0	0	0	0	4	0	8	0	3	0	8	0	1	0	0.115	0	0.01	0
68	07/01/92	61	1	dyspepsia	ODN	0	normal	0	0	0	0	8	0	70	0	33	0	80	0	8	0	0.17	0	0.01	0
72	21/10/93	41	1	ODR	GDN	0	normal	0	0	0	0	13	0	1	0	3	0	3	0	3	0	0.112	0	0.00	0
79	07/04/93	48	0	dyspepsia	OCN	0	normal	0	0	0	0	3	0	17	0	21	0	32	0	4	0	0.124	0	0.00	0
80	03/02/93	41	0	urt. dyspepsia	GDN	0	normal	0	0	0	0	3	0	11	0	8	0	27	0	7	0	0.2	1	0.06	0
82	24/11/93	42	1	GCA	OON	0	normal	0	0	0	0	3	0	25	0	13	0	25	0	4	0	0.130	0	0.00	0
86	11/01/85	36	0	dyspepsia	ODN	0	normal	0	0	0	0	7	0	0	0	0	0	0	0	7	0	0.128	0	0.08	0
87	30/03/84	73	0	GDA	GDN	0	normal	0	0	0	0	72	0	3	0	4	0	11	0	3	0	0.173	0	0.81	0
91	13/01/84	17	0	dyspepsia	ODN	0	normal	0	0	0	0	12	0	40	0	18	0	41	0	2	0	0.108	0	0.01	0
94	13/02/82	38	0	dyspepsia	GDN	0	normal	0	0	0	0	21	0	72	0	15	0	38	0	1	0	0.774	1	0.07	0
96	11/03/90	33	1	dyspepsia	ODN	0	normal	0	0	0	0	19	0	43	0	27	0	54	0	1	0	0.188	0	0.00	0
97	08/01/86	42	1	CDA	GDN	0	normal	0	0	0	0	3	0	3	0	4	0	11	0	0	0	0.148	0	0.02	0
98	22/03/92	34	1	dyspepsia	GDN	0	normal	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0.122	0	0.00	0

Table 6: Sera of population II
In relation to the presence of Hp (culture and/or anamatopathologically) and ulcer

				Sensibility	Specificity
In relation to Hp+ and DU/GU that is: 34Hp+/U+	NS/IbA-	HspA malE	VS=100	44.1% (15/34)	100% (31/31)
			VS=50	52.9% (18/34)	100% (31/31)
			VS=20	64.7% (22/34)	73.8% (25/31)
		NOG	VS=100	94.1% (32/34)	96.8% (30/31)
			VS=80	94.1% (32/34)	93.6% (29/31)
			VS=60	100% (34/34)	90.3% (28/31)
		PBS	VS=100	82.4% (28/34)	93.6% (29/31)
			VS=80	94.1% (32/34)	93.6% (29/31)
			VS=60	97.1% (33/34)	83.9% (26/31)
		CLF Sero	VS=0.30	82.4% (28/34)	96.8% (30/31)
		Pylori Stat		94.1% (32/34)	90.3% (28/31)
		Cobas Core		100% (34/34)	93.6% (29/31)

Table 7: Sera of population II
In relation to the presence of Hp (culture and/or anamatopathologically)

In relation to Hp+: -34 DU/GU -27 GNU that is: 61 Hp+ 31 Hp-	N6ΠbA-	HspA malE	VS=100	Specificity 45.9% (28/61)	Sensibility 100% (31/31)
			VS=50	59% (36/61)	100% (31/31)
			VS=20	80.7% (45/61)	73.8% (25/31)
		NOG	VS=100	95.1% (58/61)	96.8% (30/31)
			VS=80	95.1% (58/61)	93.6% (29/31)
			VS=60	100% (61/61)	90.3% (28/31)
		PBS	VS=100	85.3% (52/61)	93.5% (29/31)
			VS=80	93.4% (57/61)	93.6% (29/31)
			VS=50	95.7% (59/51)	83.9% (26/31)
		JLP Sero	VS=0.30	78.7%(48/61)	96.8% (30/31)
		Pylori Stat		93.4% (57/61)	90.3% (28/31)
		Cobas Core		93.3%(60/61)*	93.6% (29/31)

*Serum + VS

Table 5: Sera of population II
In relation to the presence of Hp (culture and/or anamatopathologically) and the absence of an ulcer

				Specificity	Sensibility
In relation to Hp+ and GNU that is: 27 Hp+/U-	NAP-NAP	HspA malE	VS=100	48.2% (13/27)	100% (31/31)
			VS=50	66.7% (18/27)	100% (31/31)
			VS=20	85.2% (23/27)	73.8% (25/31)
		NOG	VS=100	96.3% (26/27)	96.8% (30/31)
			VS=60	93.6% (26/27)	93.6% (29/31)
			VS=60	100% (27/27)	90.3% (28/31)
		PBS	VS=100	88.9% (24/27)	93.6% (29/31)
			VS=60	92.6% (25/27)	93.6% (29/31)
			VS=60	96.3% (26/27)	83.9% (26/31)
		JLP Sero	VS=0.30	74.1% (20/27)	96.8% (30/31)
		Pylori Stat		92.6% (25/27)	90.3% (28/31)
		Cobas Core		96.3% (26/27)	93.6% (29/31)

The place of serology

Serology is placed at 2 levels:

- Very sensitive serology: for the purpose of detecting the presence of the bacterium in young subjects complaining of epigastric pains.

If the serology turns out to be negative, the subject will not have to suffer endoscopy or a biopsy and another cause for his pains will be sought.

- Risk-specific serology: this involves demonstrating the risk of having a serious infection with Helicobacter pylori, that is an ulcer, a cancer or a gastric lymphoma (MALT lymphoma).

- either using a molecule which is specific for the risk in question

- or using a risk-specific threshold (threshold value which is higher in subjects which are at risk than in subjects which are not at risk).

This specific serology can be employed to screen the general population and thus to detect cancers and lymphomas which are associated with Helicobacter pylori and which would not be detected because of a lack of symptoms. (Only subjects which complain of pain will consult a gastroenterologist).

The response to the sensitivity issue is good.

Table 9: Mean and standard deviation of the A.U.'s in the 3 groups of patients

		Hp-(n=31)	Hp+/U-(n=27)	Hp+/U+(n=34)
Hsp A	mean	10.61	775.72	770.32
	standard deviation	8.81	1312.56	1666.52
NSf13A-(NOG)	mean	17.16	895.50	944.35
	standard deviation	26.69	918.57	915.27

Table 10: Mean and standard deviation of the A.U.'s in terms of gastric histology

Intensity		Atrophy			Inflammation				Activity		
		Hsp A	NOG	P.Stat	Cag A	Hsp A	NOG	P.Stat	Hsp A	NOG	P.Stat
0	Mean (standard deviation)								977 2052	712 680	0.31 0.08
1	Mean (standard deviation)	410 1004	412 390	0.28 0.07	91 122	437 609	877 400	0.30 0.00	479 1117	938 878	0.32 0.09
2	Mean (standard deviation)	423 984	730 707	0.30 0.08	188 200	[illegible] 1055	[illegible] 780	0.31 0.09	733 1382	798 753	0.31 0.07
3	Mean (standard deviation)	1321 2059	1403 1012	0.38 0.08	884 807	[illegible] 1742	[illegible] 1132	0.43 0.08	1302 1028	1402 1174	0.38 0.12

01 Hp+ :

Distribution	Atrophy	Inflammation	Activity
0	0	0	15
1	10	21	25
2	26	33	12
3	22	7	9
4	1	0	0

Correlation between the intensity of the gastritis and the antibody levels

The gastritis is defined by 3 parameters:

- Atrophy (represented by the first figure after G); its intensity is marked from 1 to 4.
- The global inflammation corresponds to infiltration with neutrophilic polynuclear cells and with monocytes; (represented by the second figure after the G). Its intensity is marked from 1 to 3.
- Activity corresponds to the number of neutrophilic polynuclear cells (represented by the third figure after the G); its intensity is marked from 0 to 3. Some folicular forms are marked F.

Normally, the following correlation can be observed:

The activity correlates very well with Helicobacter pylori.

The inflammation correlates well with Helicobacter pylori.

The means of the titres observed in each group have therefore been calculated in terms of these 3 parameters and their intensity.

Interpretation of the results:

Use of a t test makes it possible to demonstrate whether a difference between 2 observed means is significant or not with a 5% risk.

The hypothesis on which the t test is based is the equality of variances, demonstrated by an F test (Fisher test).

Since some variances are not equal, it is not therefore possible to compare all the means with each other.

By comparing the means, when possible, it has been possible to demonstrate whether the differences between the different groups are significant or not.

- Significant difference:

Between the means of "2" and "3" for HspA and NOG in the "Inflammation" group.

- <u>Non-significant difference:</u>

With regard to activity, no significant differences were demonstrated between the different intensity levels:

- <u>HspA</u>:

no significant difference between levels
0 and 2
0 and 3
1 and 2
1 and 3
2 and 3

- <u>NOG</u>:

no significant difference between levels
0 and 1
0 and 2
1 and 2
1 and 3
2 and 3.

It is nevertheless possible to observe a tendency for the titres to increase in dependence on the intensity of the gastritis:

- <u>with regard to atrophy</u>, the means double, for HspA and for the NOG extract of the aflagellate strain, when passing from level 1 to 2 and from level 2 to 3.
- <u>with regard to inflammation</u>, the means double when passing from level 1 to 2.

The numbers in each group are relatively low (in each case <30) for drawing conclusions with regard to statistically significant differences.

Table 11: Means of the A.U.'s in terms of gastric histology

Intensity	For HP+/U+	Atrophy HspA	Atrophy NOG	Atrophy P.Stat	Inflammation HspA	Inflammation NOG	Inflammation P.Stat	Activity HspA	Activity NOG	Activity P.Stat
0	Mean (standard deviation)							1292 (2610)	875 (897)	0.32 (0.09)
1	Mean (standard deviation)	121 (116)	328 (218)	0.25 (0.05)	438 (759)	311 (451)	0.30 (0.08)	833 (1298)	1818 (1050)	0.34 (0.10)
2	Mean (standard deviation)	304 (507)	793 (784)	0.32 (0.09)	883 (1820)	848 (813)	0.33 (0.09)	219 (329)	827 (835)	0.32 (0.09)
3	Mean (standard deviation)	2004 (2850)	1722 (1080)	0.41 (0.07)	2133 (1989)	1194 (1000)	0.43 (0.09)	911 (1502)	1316 (1040)	0.38 (0.12)

34 Hp+/U+:

Distribution	Atrophy	Inflammation	Activity
0	0	0	8
1	7	10	13
2	17	19	8
3	9	5	6
4	1	0	0

Sera able to exhibit cross reactions

2 types of sera were employed.

20 sera (10 anti-Legionella + and 10 anti-Chlamydia +) being able to exhibit cross reactions with HspA, because these 3 bacteria possess heat shock proteins which are very akin to each other.

3 anti-Campylobacter positive sera, in order to demonstrate cross reactions with the flagellate strain N6 which would disappear with the aflagellate strain N6flbA-. It is very difficult to obtain anti-Campylobacter positive sera; this is the reason for there only being 3 sera.

HspA does not exhibit any cross reaction, either with the 10 anti-Legionella positive sera or with the 10 anti-Chlamydia positive sera.

While some of these sera have positive titres of anti-Helicobacter pylori antibodies, both with the flagellate strain and with the aflagellate strain, the clinical context of these sera is not known.

Table 12: Sera which are able to exhibit cross reactions

Legionella +	Titre	NS	VS=100	NSΠBA-	VS=60	HspA	VS=100
A	P2 P3 =256	0	0	4	0	47	0
B	P4 P5 =64	>928	1	641	1	42	0
C	P2 P3 =128	212	1	87	1	68	0
D	P2 P3 =64	70	0	19	0	15	0
E	P1=256 /P2=512	>928	1	259	1	258	1
F	P2 P3 P4 P5 =128	322	1	121	1	41	0
G	P1=512 /P6=1024	>928	1	193	1	121	1
H	P4 P5 =64	>828	1	479	1	18	0
I	P2=128 /P3=64	33	0	17	0	25	0
J	P2=256 /P3=128	16	0	8	0	32	0

Chlamydia +	Titre	NS	VS=100	NSΠBA-	VS=60	HspA	VS=100
A	256	6	0	8	0	25	0
B	256	7	0	9	0	34	0
C	64	635	1	280	1	39	0
D	256	357	1	225	1	18	0
E	32	>928	1	855	1	19	0
F	128	>828	1	783	1	27	0
G	32	115	1	55	0	15	0
H Twar	16	19	0	10	0	14	0
I	32	>928	1	592	1	>828	1
J Twar	64	610	1	280	1	44	0

Campylobacter +		NS	VS=100	NSΠBA-	VS=60	HspA	VS=100
A		35	0	28	0	17	0
B		13	0	4	0	27	0
C		50	0	68	1	89	0

CONCLUSION

HspA malE

It is still not possible to use this molecule on its own since it also lacks sensitivity, but it could be of interest if it is associated with other molecules.

It nevertheless carries a risk of cross reactions due to the substantial conservation of these heat shock proteins between the different bacterial species.

N6flbA-

This aflagellate variant appears to be of great interest; the sensitivity and specificity which were obtained with serum population II demonstrate a very favourable efficacy.

N6

For the time being, the flagellate strain appears to be of interest. However, the cross reactions relating to the flagellum have only been studied to a limited extent due to the difficulty of obtaining sera which are well documented with regard to Campylobacter serology.

JLF test

A serological test based on an aqueous (PBS) extract of several strains of Helicobacter pylori was developed. This test appears to be very efficacious.

A NOG extract of the aflagellate variant was used to test serum population I.

87 sera, which were documented only from the bacteriological and anatomopathological points of view, were tested with the aflagellate bacterial extract.

A serum is positive if the culture is positive or if the anatomopathology and the rapid urea test are positive.

A serum is negative if the 3 tests (culture, anatomopathology and rapid urea test) are negative.

A sensitivity of 90.3% (28/31) is found together with a specificity of 71.4% (40/56).

Of 16 sera which are falsely positive using a first test, 9 are positive either using JLF serology or using the JLF Western blot, or using both of them.

Of the 3 sera which are falsely negative using a first test, all 3 are negative either with JLF serology or with JLF Western blot, and one serum is negative with both the systems.

Table 13: 87 sera from population I tested with the n-octyl glucoside extract of the aflagellate strain

No. of serum	HspA	VS 150	JLF sero	VS 35	WB JLF	interp	WB Bioptim	Hp	WGTBA NOG	WGTBA VS+60
572	35	0	21	0	2p	-	+	0	128	[illegible]
573	11	0	46	1	3p	+	-	1	229	1
574	11	0	3	0	1p	-	-	0	8	0
575	0	0	83	[illegible]	3p	[illegible]	-	0	166	[illegible]
576	121	0	19	0	3p	[illegible]	+	0	246	[illegible]
577	0	0	1	0	0	-	-	0	3	0
578	6	0	4	0	0	-	-	0	24	0
579	2630	1	114	1	3p	+	-	1	>464	1
580	721	1	125	1	4p	+	-	1	>464	1
581	0	0	2	0	0	-	-	0	2	0
582	0	0	2	0	1p	-	-	0	8	0
583	0	0	3	0	2p	-	-	0	27	0
584	36	0	1	0	2p	-	-	0	12	0
585	2114	1	125	1	4p	+	-	1	>464	1
587	19	0	2	0	2p	-	-	0	11	0
588	1386	1	58	1	3p	+	-	1	>464	1
589	323	1	3	0	4p	[illegible]	+	0	>464	[illegible]
591	4	0	4	0	2p	-	-	0	9	0
592	6	0	0	0	2p	-	+	0	9	0
593	44	0	28	0	3p	[illegible]	-	1	3	[illegible]
595	76	0	76	1	4p	+	+	1	>464	1
597	0	0	0	0	0	-	-	0	9	0
599	49	0	125	1	4p	+	+	1	>464	1
600	0	0	3	0	0	-	-	0	3	0
601	8	0	1	0	0	-	-	0	8	0
602	0	0	0	0	0	-	-	0	0	0
605	11	0	0	0	0	-	-	0	10	0
608	5	0	5	0	0	-	-	0	9	0
609	308	1	8	0	0	-	-	0	13	0
610	2370	1	111	1	4p	+	-	1	>464	1
612	477	1	34	0	4p	[illegible]	+	0	422	[illegible]
613	46	0	0	0	0	-	-	0	3	0
618	741	1	73	1	4p	+	+	1	>464	1
617	1725	1	125	1	4p	+	-	1	286	1
618	428	1	101	1	4p	+	+	1	>464	1
621	0	0	82	1	4p	+	+	1	>464	1
622	15	0	8	0	2p	-	-	0	25	0
624	411	1	110	[illegible]	4p	[illegible]	+	0	>464	[illegible]
626	46	0	11	[illegible]	1p	[illegible]	-	1	53	[illegible]
627	0	0	48	1	1p	[illegible]	-	1	27	[illegible]
629	6	0	2	0	0	-	-	0	2	0
631	31	0	21	0	2p	-	-	0	92	[illegible]
632	0	0	3	0	0	-	-	0	22	0
633	285	1	104	1	3p	+	+	1	>464	1
634	46	0	88	1	4p	+	-	1	>464	1
636	523	1	33	0	2p	-	-	1	71	1

Table 14: 87 sera from population I tested with the n-octyl glucoside extract of the aflagellate strain

No. of serum	HspA	VS 150	JLF sero	VS 35	WB JLF	Interp	WB Bioptim	Hp	N6MBA- NOG	N6MBA- VS-60
838	822	1	38	1	3p	+	+	1	>464	1
841	0	0	6	0	1p	-	-	0	8	0
845	20	0	6	0	1p	-	-	0	20	0
847	0	0	2	0	1p	-	-	0	4	0
849	5	0	5	0	0	-	-	0	12	0
850	6	0	0	0	0	-	-	0	3	0
854	0	0	1	0	0	-	-	0	4	0
855	49	0	58	1	2p	-	-	1	220	1
856	0	0	3	0	0	-	-	0	8	0
857	363	1	105	1	4p	+	+	1	>464	1
858	0	0	8	0	1p	-	-	0	8	0
859	0	0	3	0	0	-	-	0	3	0
862	73	0	3	0	2p	-	-	0	40	0
863	25	0	21	0	2p	-	-	0	103	[illegible]
457	86	0	28	0	4p	[illegible]	-	0	96	[illegible]
465	32	0	58	1	4p	+	+	1	>464	1
469	265	1	118	1	3p	+	+	1	>464	1
470	734	1	77	[illegible]	2p	++	+	0	>464	[illegible]
471	214	1	100	1	4p	+	-	1	>464	1
472	4	0	5	0	0	-	-	0	0	0
473	1023	1	55	1	3p	+	-	1	>464	1
474	12	0	10	0	0	-	-	0	21	0
475	9	0	13	0	0	-	+	0	210	[illegible]
476	2611	1	74	1	4p	+	+	1	>464	1
478	0	0	0	0	0	-	-	0	1	0
479	175	1	9	0	4p	[illegible]	-	0	348	[illegible]
480	0	0	1	0	0	-	-	0	7	0
481	800	1	82	1	3p	+	+	1	425	1
482	0	0	1	0	0	-	-	0	8	0
483	0	0	39	1	3p	+	+	1	>464	1
484	0	0	3	0	0	-	-	0	20	0
485	0	0	1	0	0	-	-	0	11	0
486	0	0	2	0	0	-	-	0	6	0
725	0	0	7	0	0	-	-	0	186	[illegible]
730	180	1	45	[illegible]	1p	-	-	0	372	[illegible]
732	0	0	10	0	1p	-	-	0	145	[illegible]
735	0	0	30	0	2p	-	-	1	143	1
736	0	0	0	0	0	-	-	0	0	0
737	25	0	102	1	4p	+	-	1	155	1
735	2233	1	125	1	4p	+	-	1	>464	1
739	78	0	33	0	1p	-	+	0	274	[illegible]

TECHNIQUE

Plates coated with: HspA antigen at 2 $\mu g/ml$
NOG extract of NflbA and N6 at 3 $\mu g/ml$

Range: 5 range points negative control
positive control
used at 4 dilutions

Patient sera: 1/100 dilution
volume deposited: 100 μl

- Incubation: 37°C for 1 hour
- 3 washings:

Monoclonal conjugate (IgG toxo)
used at 1/32,000 for HspA
1/64,000 for N6flbA-
1/56,000 for N5
volume deposited: 100 μl

- Incubation of the conjugate: 37°C for 1 hour
- 4 washings
- Development of the enzyme reaction using OPD + substrate
30 minutes in the dark
- Termination of the enzyme reaction with H_2SO_4
- Reading of the OD at 492 nm/620 nm

Conversion of the OD's into arbitrary units (AU).

Table 15: Documented sera from population 1
47 Hp+ sera

No. of serum	Sex	Date of birth	Endos.	ANATOMICPATHOLOGY		BACTERIOLOGY			Hp	ELP Sero	VS=0.3	NOG	VS=60
				Glemsa	Histo	Gram	Urea	Cult.					
952253	1	01/10/60	G, H	0	G	1	1	1	1	1	1	>464	1
236174	1	02/05/60	G	0	G	1	1	1	1	10.42	1	216	1
974107	2	15/02/32	G (mini)	0	G	1	1	1	1	1.39	1	272	1
34812	1	10/12/52	G, B	0	G	1	1	1	1	0.82	1	452	1
229712	2	11/08/53	G	0	G	1	1	1	1	0.11	[illegible]	148	1
46511	1	17/01/70	G	0	G	1*	0	1	1	1.20	1	213	1
180334	2	14/01/59	G	0	U	1	1	1	1	0.80	1	>464	1
189005	2	23/10/25	U	0	U	1	1	1	1	0.87	1	>464	1
49860	1	06/07/64	U	0	U	1	1	1	1	2	1	>464	1
188332	1	06/11/60	G	1	G	1	1	1	1	0.23	[illegible]	394	1
195282	1	11/06/46	G	1	G	1	0	1	1	0.91	1	180	1
176859	1	24/04/50	G	1	G	1	1	1	1	1.39	1	>464	1
987890	1	13/05/58	G	1	G	1	1	1	1	0.69	1	297	1
954458	2	01/12/45	G	1	G	1	1	1	1	1.12	1	>464	1
185175	2	01/08/08	G	1	G	0	0	1	1	2.7	1	>464	1
156007	2	27/09/58	G, B	1	G	1	1	1	1	1.68	1	437	1
18310	1	19/12/63	G, B	1	G	1	1	1	[illegible]	0.38	1	45	[illegible]
215979	2	04/05/18	H, G, B	1	G	1	1	1	1	1.2	1	>464	1
25322	2	12/02/18	G	1	G	1	1	1	1	2.5	1	>464	1
26555	1	09/11/65	U, H	1	G	1	1	1	1	2.4	1	>464	1
193295	1	24/01/18	ant. bu. U	1	G	1	1	1	1	2.5	1	>464	1
237220	1	01/06/65	bulb. U	1	G	1	0	1	1	0.14	[illegible]	328	1

Legend

G = Gastritis
H = Hiatus hernia
U = Ulcer (DU = Duodenal ulcer)
(GU = Gastric ulcer)
D = Duodenitis
D/Bulb = Bulbitis
O = Oesophagitis

Table 15 bis: Documented sera from copulation I
42 Hp + sera

No. of serum	Sex	Date of birth	Endos.	ANATOMOPATHOLOGY		BACTERIOLOGY			Hp	ULP Sero.	VS=0.3	NOG	VS=60
				Glamsa	Histo	Gram	Urea	Cult.					
237191	1	06/05/42	bulb. U	1	G	1	0	1	1	1.16	1	>464	1
238683	1	13/09/30	G, bulb U	1	G	1	1	1	1	1.73	1	>464	1
79163	1	06/07/72	G	1	G	1	1	1	1	0.46	1	312	1
87851	1	15/04/41	G	1	G	1	1	1	1	0.7	1	>464	1
93773	1	12/05/43	G	1	G	0	0	1	1	1.05	1	>464	1
97478	1	04/05/65	G	1	G	1	1	1	1	0.42	1	>464	1
96436	1	06/11/74	G	1	G	1	1	1	1	0.84	1	183	1
66502	1	02/10/45	G	1	G	1	1	1	1	0.78	1	>464	1
42230	2	12/06/58	G	1	G	1	1	1	1	0.81	1	>464	1
51105	2	12/08/45	G, DU	1	G	1	1	1	1	1.1	1	>464	1
68631	1	21/02/43	G	1	G	1	1	1	1	0.8	1	214	1
79105	2	28/01/61	G, DU	1	G	1	1	1	1	1.26	1	>464	1
89121	1	28/10/59	G	1	G	1	1	1	1	0.8	1	449	1
216778	1	08/04/47	G, U	1	G/U	1	1	1	1	0.25	[illegible]	283	1
996070	1	29/01/47	G	1	Preatroph. G	1	1	1	1	0.31	1	121	1
72420	1	15/05/55	G, DU	1	G CU	1	1	1	1	1.2	1	>464	1
205110	1	10/06/61	CU	1	U	1	1	1	1	0.3	[illegible]	388	.1
62720	1	18/10/58	CU	1	U	1	1	1	1	0.68	1	>464	1
07767	2	01/10/44	CDU	1	U	1	1	1	1	1.2	1	>464	.1
205855	1	09/07/38	G, U	1	CU	1	1	1	1	0.25	[illegible]	71	.1

Legend
G - Gastritis
H - Hiatus hernia
U - Ulcer (DU - Duodenal ulcer)
(GU - Gastric ulcer)
D - Duodenitis
B/Bulb. - Bulbitis
O - Oesophagitis

Table 16: Documented sera from population I

55 Hp- sera

No. of serum	Sex	Date of birth	Endos.	HISTOPATHOLOGY		BACTERIOLOGY			Hp	ELP sero.	VS=0.3	NOD	VS=60
				Glomus	Histo	Gram	Urea	Cult.					
79476	1	23/06/31	G	0	Ulcerated adenoma	0	0	0	0	0.02	0	8	0
75439	2	14/05/32	G	0	G	0	0	0	0	1.19	[illegible]	>464	[illegible]
97286	2	03/01/37	G	0	G	0	0	0	0	0.45	[illegible]	60	[illegible]
68053	1	02/05/48	G	0	G	0	0	0	0	1.02	[illegible]	304	[illegible]
71300	2	14/10/63	G	0	G	0	0	0	0	0.89	[illegible]	>464	[illegible]
944950	1	01/10/54	G	0	G	0	0	0	0	0.04	0	4	0
967659	2	20/01/48	G	0	G	0	0	0	0	0.01	0	5	0
985409	2	26/07/20	min. U	0	G	0	0	0	0	0.21	0	69	[illegible]
985551	2	18/08/09	G, U, B	0	G	0	0	0	0	0.05	0	14	0
992025	1	22/03/32	G	0	G	0	0	0	0	0.07	0	65	[illegible]
998792	2	11/04/44	G	0	G	1	0	0	0	0.08	0	20	0
16479	1	13/07/93	RAS	0	G	0	0	0	0	0.02	0	0	0
77183	2	24/08/14	G, U	0	G	0	0	0	0	0.03	0	8	0
77566	1	25/01/32	G	0	G	0	0	0	0	0.01	0	22	0
991337	1	24/10/60	G	0	Hyperplasia	0	0	0	0	0.07	0	28	0
78471	2	13/12/15	G	0	Gelatinat. retepl.	0	0	0	0	0.07	0	108	[illegible]
83350	1	10/07/21	cicat. U	0	min. C	0	0	0	0	0.37	[illegible]	>464	[illegible]
936515	2	05/06/81	RAS	0	min. C	0	0	0	0	0.02	0	0	0
991386	2	22/01/71	G	0	min. C	1	0	0	0	0.17	0	3	0
6130	1	05/05/72	G	0	min. C	0	0	0	0	0.08	0	0	0
81415	1	23/08/31	G	0	min. C	0	0	0	0	0.03	0	5	0
82175	1	13/01/49	cicat. U	0	min. C	0	0	0	0	0.03	0	34	0
70652	1	01/08/18	G, U	0	gast. hypotrophia	0	0	0	0	0.04	0	10	0
89819	2	16/02/42	Normal	0	Normal	0	0	0	0	0.88	[illegible]	>464	[illegible]
942184	2	09/02/87	G	0	Normal	0	0	0	0	0.1	0	52	0
981000	2	10/10/47	G	0	Normal	0	0	0	0	0.08	0	18	0
1613	1	11/01/20	G, B, D	0	Normal.	0	0	0	0	0.88	[illegible]	195	[illegible]

Table 16bis: Documented sera from population 1
55 Hp- sera

No. of serum	Sex	Date of birth	Endos.	ANATOMOPATHOLOGY		BACTERIOLOGY			Hp	ELISA Sero.	VS=0.3	NOG	VS=60
				Oliensa	Histo	Gram	Urea	Cult.					
984978	2	23/04/29	GU	0	Normal	0	0	0	0	0	0	3	0
58767	2	19/12/93	RAS	0	Normal	0	0	0	0	0.08	0	0	0
79861	2	26/07/63	G, O	0	Normal	0	0	0	0	0.08	0	16	0
85290	2	26/04/63	RAS	0	Normal	0	0	0	0	0.01	0	2	0
91423	1	13/02/39	RAS	0	Normal	0	0	0	0	0.03	0	0	0
93252	2	26/08/85	RAS	0	Normal	0	0	0	0	0.09	0	10	0
94430	1	06/04/62	RAS	0	Normal	0	0	0	0	0.13	0	>464	
090363	2	03/06/36	G, B	0	Normal +/-	0	0	0	0	0.18	0	42	0
87467	1	07/10/50	GDU	0	U	0	0	0	0	0.02	0	60	0
239C85	1	05/02/45	bulb., GU	0	U	0	0	0	0	0.03	0	73	
3473	1	06/02/46	G, U	1	G	0	0	0	0	1.01		>464	
70605	1	14/05/83	bulb. U	1	G	0	0	0	0	0.56		>464	
83721	1	21/01/95	G	1	G	0	0	0	0	0.61		245	
90169	1	18/04/38	G, B	1	G	0	0	0	0	1.15		>464	
91C81	2	08/01/45	G, O	1	G	0	0	0	0	1.8		>464	
43127	1	24/02/41	G	1	G	0	0	0	0	1.15		>464	
928133	2	25/03/71	G	1	G	0	0	0	0	0.03	0	3	0
9128	1	08/03/77	G	1	G	0	0	0	0	0.01	0	0	0
974895	1	11/05/38	B, G	1	G	0	0	0	0	0.08	0	2	0
26697	1	23/08/44	H, O, U	1	G	0	0	0	0	0.21	0	8	0
78414	1	06/02/21	G, B, U	1	G	0	0	0	0	0.02	0	5	0
78451	1	26/11/45	G	1	G	0	0	0	0	0.02	0	19	0
79500	1	01/03/50	Oesoph. U	1	G	0	0	0	0	0.01	0	3	0
79880	1	02/01/74	GU, B	1	G	0	0	0	0	0.06	0	5	0
416	1	18/02/71	O, G	1	min o	0	0	0	0	0.03	0	1	0
74548	1	25/02/45	GDU	1	U	0	0	0	0	0.38		371	
99538	1	02/04/58	bulb. U.	1	U	0	0	0	0	1.08		>464	
98953	2	19/12/16	U	1	U	0	0	0	0	0.62		>464	

Table 17: Documented population from population I

55 Hp- sera

42 Hp+ sera

5

	SENSITIVITY	SPECIFICITY
JLF sero	85.7% (36/42)	70.9% (39/55)
NOG 60	97.5% (41/42)	61.8% (34/55)

EXTRACTION PROTOCOLS USING THE AFLAGELLATE STRAIN N6flbA-.

Quantity supplied: 800 mg of bacteria collected using PBS and centrifuged.

3 extractions tested.

EXTRACTIONS OF THE AFLAGELLATE STRAIN

	Glycine extraction	n-octyl glucoside extraction	PBS extraction
Recovery	PBS	0.01M PBS	PBS, pH 7.4
Washing	Twice in PBS; 8000 rpm/12 min	Twice in PBS; 8000 rpm/12 min	
Extraction	0.2M acid glycine buffer, pH 2.2, for 15 min and at room temperature gentle agitation 100 mg (wet weight) per 2.5 ml	PBS containing: 1% n-octyl glucoside, pH 7.2 (Sigma Chemical Co.), for 20 min at room temperature	Vortex for 1 min.
Centrifugation	11,000 g for 15 min	23,500 g for 20 min	5,000 g for 10 min
Neutralization	1M NaOH		
Dialysis	PBS, pH 7.2, for 24 h at +4°C cut-off: 10,000	PBS, pH 7.2, for 24 hours at +4°C cut-off: 10,000	PBS, pH 7.2, for 24 h at +4°C cut-off: 10,000
Storage	determination of the concentration storage at -20°C	removal of the insoluble particles storage at -20°C	determination of the concentration storage at -20°C

SDS PAGE ON DIFFERENT EXTRACTS OF THE AFLAGELLATE STRAIN N6 FLBA-

Well No.	Sample type	Concentration µg/ml	Sample volume/ buffer volume	Volume loaded
1	MW standard			
2	Glycine extract	202.9	5 + 5/190	10
3			60/60	60
4	n-octyl glucoside extract	874	51/39	60
5				
6	PBS 1 extract	539.2		
7			50/20	60
8	PBS 2 extract	77.9		
9			60/20	60
10	MW standard			
11	Glycine extract pellet	2770.7	5 + 5/190 20/20	10 20
12				
13	Glucoside extract pellet	972.9	40/40	60
14				
15	Sedimented glycine extract	309.3	60/20	50
16				
17	HspA Mal E	3000		
18			20/20	20
19				
20	Kaleidoscope			20

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